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Expression Pattern of GDNF, *c-ret*, and GFRαs Suggests Novel Roles for GDNF Ligands during Early Organogenesis in the Chick Embryo

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We have cloned a partial cDNA of chicken glial cell line-derived neurotrophic factor (GDNF) and systematically examined its expression pattern as well as that of GDNF-binding components (GDNF family receptor alpha-1 and 2: GFRα-1 and 2) and a common signal transduction receptor (*c-ret* protooncogene: RET) during very early developmental stages. In addition, we also examined the expression pattern of an apparent avian-specific binding component, GFRα-4. The cloned chicken cDNA for GDNF had approximately 80% homology to mammalian counterparts. The expression of GDNF mRNA occurred in many spatially and temporally discrete regions such as the intermediate mesoderm, the floor plate of the spinal cord, pharyngeal endoderm contacting the epibranchial placodes, distal ganglia of cranial nerves, subpopulations of mesenchyme cells in the craniofacial region, and in the mesodermal wall of the digestive tract. Both a GDNF receptor signal transduction component (RET) and a binding component (GFRα-1 or GFRα-2) were independently expressed in nearby interacting tissues such as the somites, peripheral and central nervous system, and mesenchyme cells in the craniofacial region. These observations suggest that possible combinations of novel unidentified receptors acting with RET or with GFRαs may mediate GDNF-derived signals and indicate that GDNF or other family members may have previously unidentified actions in early organogenesis in the chick embryo.

Key Words: **GDNF; *c-ret;* GFRα; somite; floor plate; placode; cranial ganglia; *in situ* hybridization; chick embryo.**

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) was originally identified as a potent survival factor for dopaminergic neurons *in vitro* (Lin *et al.*, 1993). Subsequently, structurally related molecules, namely neurturin (NTN; Kotzbauer *et al.*, 1996), persephin (PSP; Milbrandt *et al.*, 1998), and artemin (ART; Baloh *et al.*, 1998b), have been identified and now compose a subfamily within the TGFβ superfamily of molecules. A generally accepted model of signal transduction by molecules of the GDNF family, which has been mainly derived from *in vitro* studies, is that the ligands employ a multicomponent receptor system in which a glycosylphosphatidylinositol (GPI)-linked cell surface receptor (GDNF family receptor alpha: GFRα) first binds a ligand, after which the ligand/GFRα complex binds to and activates a receptor tyrosine kinase, *c-ret* (RET). Four structurally related binding receptors, GFRα-1, 2, 3, and 4 have been isolated and are specific receptors for GDNF, NTN, ART, and PSP, respectively (Jing *et al.*, 1996, 1997; Treanor *et al.*, 1996; Baloh *et al.*, 1997, 1998a,b; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997; Widenfalk *et al.*, 1997; Enokido *et al.*, 1998; Naveilhan *et al.*, 1998; Thompson *et al.*, 1998; Worby *et al.*, 1998). However, the presence of RET modifies the specific binding between a ligand and GFRα. For example, GDNF can bind to GFRα-2 and 3 in the presence of RET, although the GDNF/GFRα-3 complex does not necessarily activate RET (Baloh *et al.*, 1997; Creedon *et al.*, 1997; Sanicola *et al.*,

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1997; Suvanto *et al.*, 1997; Worby *et al.*, 1998). Detailed receptor expression studies in mammals, however, indicate that the predicted combinations of receptors necessary to mediate signal transduction are not always observed (Nosrat *et al.*, 1997; Trupp *et al.*, 1977; Golden *et al.*, 1998; Yu *et al.*, 1998). For example, Schwann cells surrounding the axons of motoneurons express GFRα-1 but not RET (Trupp *et al.*, 1997). A mismatch between the expression of NTN receptor components has also been observed in the nervous system (Widenfalk *et al.*, 1997). Two different explanations have been proposed to explain signal transduction in the absence of one or the other receptor components: (a) unidentified novel receptors and (b) a *trans*-action model in which, for example, Schwann cell-derived GDNF binds to GFRα-1 released from the surface of Schwann cells and this complex then binds to RET expressed on motoneuron axons (Trupp *et al.*, 1977).

Because GDNF and its receptors are involved in the survival of dopaminergic neurons and motoneurons (Henderson *et al.*, 1994; Beck *et al.*, 1995; Oppenheim *et al.*, 1995; Tomac *et al.*, 1995; Yan *et al.*, 1995), cell types which are affected in specific neurodegenerative diseases, much attention has been focused on the neurotrophic survival effects of GDNF. However, the embryonic expression pattern of GDNF and GDNF receptor components suggests multiple roles in proliferation, differentiation, and inductive interactions during early development (Pachnis *et al.*, 1993; Schuchardt *et al.*, 1995; Hellmich *et al.*, 1996; Nosrat *et al.*, 1996; Suvanto *et al.*, 1996; Wright and Snider, 1996; Luukko *et al.*, 1997, 1998; Nosrat *et al.*, 1997; Widenfalk *et al.*, 1997, 1998). The deletion of the GDNF gene in the mouse embryo results in the absence of enteric neurons and failure of development and branching of the ureteric bud, and therefore absence of the kidney (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sánchez *et al.*, 1996) and RET and GFRα-1-deficient mice exhibit a phenotype similar to mice lacking GDNF (Schuchardt *et al.*, 1994; Cacalano *et al.*, 1998; Enomoto *et al.*, 1998).

To gain a better understanding of the role of GDNF and its receptor components during early embryonic development, it is important to first systematically describe the expression pattern of these factors. Moreover, an analysis of expression patterns in very young embryos may shed light on the significance of temporal mismatches in the expression of ligand and receptor components. In later developmental stages, as well as in adult animals, the situation is often more complex in that, for example, soluble forms of systemically available receptor-ligand complexes must be considered (e.g., Ip *et al.*, 1992; Davis *et al.*, 1993). By contrast, at early developmental stages, all receptor components and the corresponding ligand must be expressed within closely interacting tissues. If a particular combination of a ligand and receptor components (e.g., GDNF, RET, and GFRα-1) is not observed in a spatially and temporally restricted region where tissue interactions such as induction and epithelial-mesenchyme transition occur, then it is more likely that novel receptor components or ligands may be involved. The early development of the chick embryo is well defined and tissue interactions have been extensively studied using experimental approaches. Accordingly, we have examined the expression pattern of GDNF and its receptor components, RET and GFRα-1, 2, and 4 in the chick embryo as a beginning attempt to understand their role(s) in early organogenesis. Efforts to examine development following perturbations of GDNF and its receptor components are in progress.

MATERIALS AND METHODS

Fertilized eggs were purchased from Daiichi Farm (Gumma, Japan). The eggs were incubated in the laboratory at 38°C and 60% relative humidity until they reached the desired stages. Embryos were staged using the Hamburger and Hamilton (1951) stage series.

Isolation of Chick GDNF

Reverse-transcriptase polymerase-chain reaction (RT-PCR) was used to isolate the chicken homologue of GDNF. Poly(A)$^+$ RNA was collected from chick embryos at stages 10–11 using the Micro Fast Track kit (Invitrogen) following the manufacturer's instructions. This RNA was copied into first-strand DNA using a reverse-transcriptase PCR kit (Perkin Elmer Cetus). To isolate the chick homologue of GDNF gene, we used 5′-TTATGGGATG-TCGTGGCTGTCT-3′ and 5′-ACATCCACACCGTTTAGCG-3′ oligonucleotide primers. These primers correspond to nucleotide positions 56–77 and 661–679 of rat GDNF (GenBank Accession Number L15305), respectively. Cycling parameters were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 28 cycles. The PCR products were cloned using the TA Cloning kit (Invitrogen). Both strands of several clones were sequenced using the ABI 373 automated sequencer.

Isolation of Chick GFRα-1 and GFRα-2

Total RNA was isolated from Embryonic Day (E) 6 spinal cord using RNeasy kit (QIAGEN) and then the first-strand DNA was reverse-transcribed using the T-primed first-strand kit (Pharmacia Biotech). PCR was performed using parameters of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles using *Taq* polymerase (Perkin-Elmer Ceteus). The primers were 5′-TCCTC-GCGCTCCTCTACTTG-3′ and 5′-AGCCCCGAGTAAGC-GAGGAG-3′ for GFRα-1, and 5′-CAAGAGGGGCATGAG-AAAG-3′ and 5′-CATTGCCGAAGGCTTGGA-3′ for GFRα-2. The PCR products of the expected size were ligated into pGEM-T Easy vector (Promega) and sequenced to verify them as chicken GFRα-1 and GFRα-2.

Probes Labeling

A plasmid encoding the extracellular domain of the chick *c-ret* protooncogene was a generous gift from Dr. F. Costantini. Digoxigenin-labeled riboprobes were synthesized from the linearized template with *Sac*I using T3 RNA polymerase (antisense probe) and *Kpn*I-linearized template using T7 RNA polymerase (sense control probe). Plasimid containing GFRα-4 was a kind gift from Dr. J. X. Comella. The sense probe was synthesized with T7 polymerase from *Sal*I-linearized template and the antisense probe was from *Sac*II-linearized template with SP6 RNA polymerase.

The GFRα-1 digoxigenin-labeled riboprobes were transcribed from *Apa*I-linearized template using SP6 RNA polymerase (antisense probe) and *Spe*I-linearized template using T7 RNA polymerase (sense probe). The GFRα-2 riboprobes labeled with digoxigenin were synthesized from the template linearized with *Apa*I using SP6 RNA polymerase (antisense probe) and *Spe*I-linearized template using T7 RNA polymerase.

For GDNF probes, a portion of the larger PCR product was reamplified using primers 5′-GTTCGATGAGGTAGTGGACT-3′ and 5′-GTCATCGTCAAAGGCTGTGG-3′ and subcloned into pGEM-T Easy vector (Promega). This probe does not discriminate between short and long forms of GDNF transcripts. The *Apa*I-linearized plasmid was used to transcribe digoxigenin-labeled riboprobes with SP6 RNA polymerase (antisense) and *Sal*I-linearized template for control probe with T7 RNA polymerase.

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization was carried out essentially as described by Wilkinson (1992). However, we omitted the hydrogen peroxide treatment and used 0.5% CHAPS and 0.1% Tween 20, instead of 1% SDS, in the hybridization and washing solutions. Hybridization temperatures were 70°C for all probes, except GDNF (65°C). For GDNF *in situ* hybridization, embryos were washed twice with 0.1 M maleic acid buffer at 65°C for 30 min after the last wash with 2× SSC and 50% formamide to reduce background levels. To obtain the best signal to noise ratio with the GDNF probe, treatment with relatively high concentrations of proteinase K was necessary, compared to that used for the other probes. Observations in whole-mount preparations were derived from 20–30 embryos for each probe at each stage. Five to 10 embryos were selected and sectioned (30 μm thickness) to better localize hybridization signals.

RESULTS

Isolation of Chick GDNF

Because the cDNA for chicken GDNF has not previously been reported, we begun with the isolation of chicken GDNF cDNA by reverse-transcriptase polymerase-chain reaction.

Two PCR products were obtained. These were sequenced and analyzed thorough BLAST service in GenBank. The two PCR products were 645 and 546 bp in size and have significant homology to the rat, mouse, and human GDNF cDNA. By comparing the two products, we found that the smaller one had a 99-bp deletion from the larger one, suggesting that the smaller one is an alternative splice variant (Fig. 1A). A short form of GDNF transcript, in which a deletion was located in the prodomain of the precusor protein sequence, has also been reported in rat and human (Suter-Crazzolara and Unsicker, 1994; Choi-Lundberg and Bohn, 1995; Cristina *et al.*, 1995; Springer *et al.*, 1995; Grimm *et al.*, 1998). In the human GDNF gene, deletion in a short form of the transcript corresponds to the 3′ end of exon 2 (Grimm *et al.*, 1998). Detection of long and short forms of GDNF mRNA in the chick embryo suggests that the chicken GDNF gene may have a genomic structure similar to that of mammals.

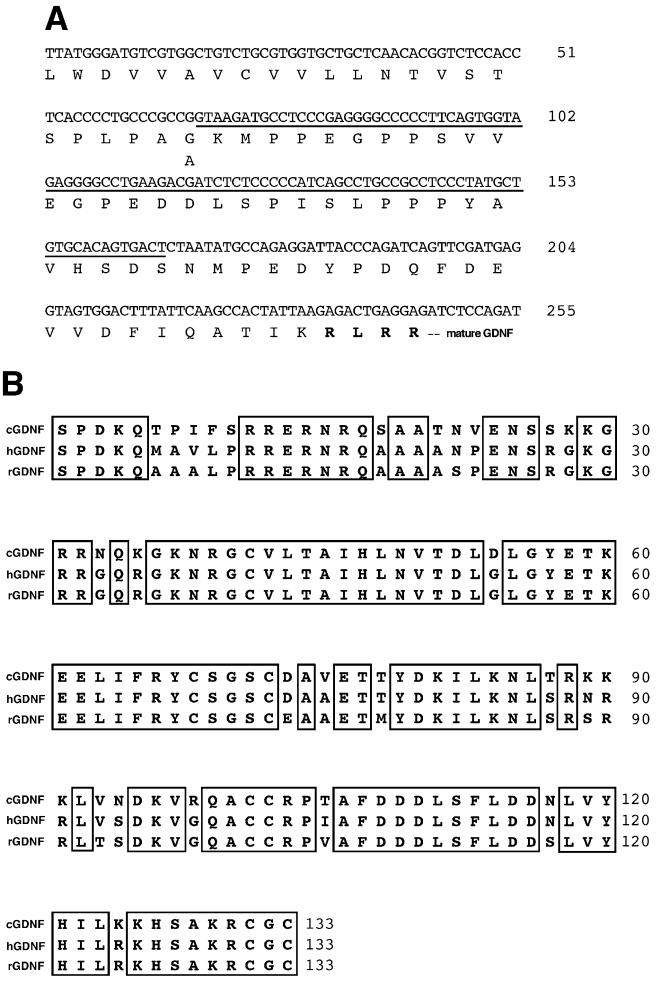

FIG. 1. The predicted amino acid sequence of chicken GDNF. (A) The top row shows chicken GDNF cDNA and the bottom row is the deduced amino acid sequence of precursor GDNF. Only the beginning of the sequence to the consensus proteolytic cleavage site for processing (R-X-X-R), which is in bold, is depicted. The underlined region is deleted in the short form of GDNF cDNA. Due to the deletion, the glycine residue is changed into an alanine. (B) Alignment of mature protein sequences of chick (c), human (h), and rat (r) GDNF using the Clustal method of multiple sequence alignment. Only those portions of human and rat GDNF sequences corresponding to the cloned partial sequence of chicken GDNF are shown. Residues that are identical among the three species are boxed.

The predicted amino acid sequence of the mature region in cloned GDNF had approximately 78 and 82% similarities to corresponding mature regions of rat and human GDNF, respectively (Fig. 1B). Although the chicken GDNF cDNAs are PCR products and not full length, they covered almost 100% of the coding regions of their human and rodent counterparts. The cDNA sequences of the long and short forms of chicken GDNF are deposited in GenBank

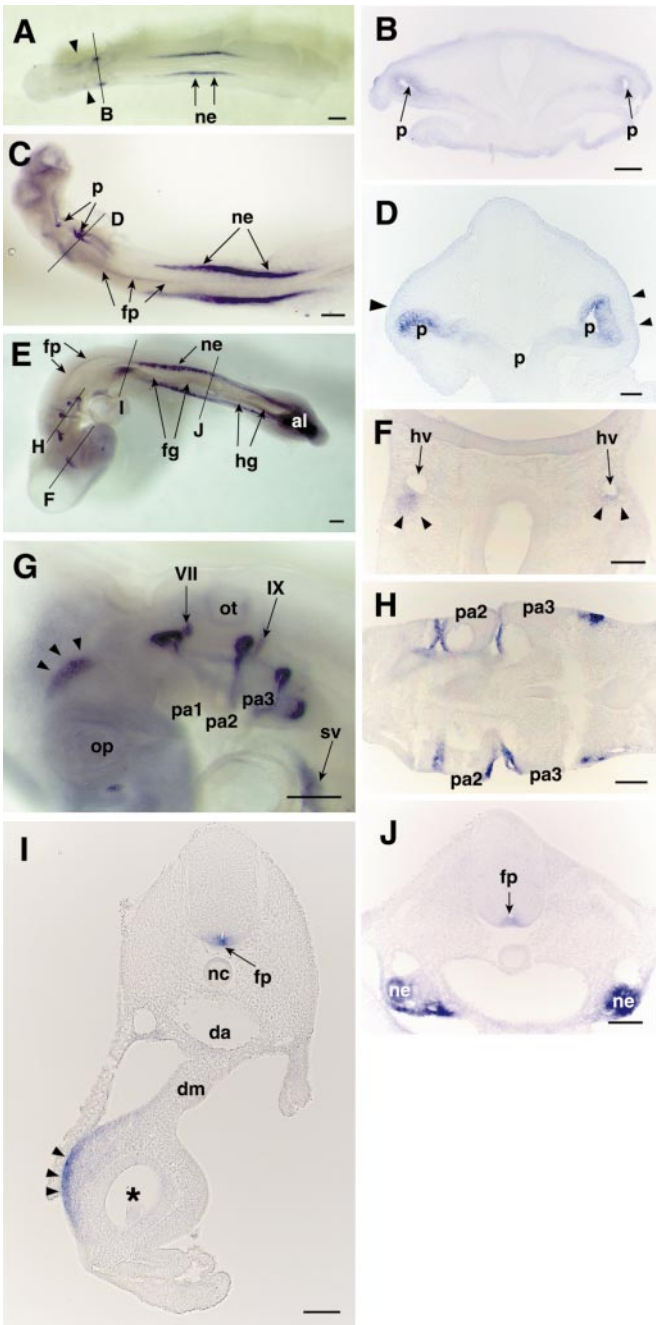

FIG. 2. Early embryonic expression of GDNF in whole-mount *in situ* hybridization (A, C, E, and G) or 30-μm sections (B, D, F, H, I, and J). Thin lines in A, C, and E indicate the approximate levels of corresponding sections shown in B, D, F, H, I, and J. (A) Dorsal view of an embryo at stage 9. Discrete focal expression of GDNF in the prospective first pharyngeal pouches (arrowheads). Expression in the pronephrogenic intermediate mesoderm (ne) is indicated by arrows. (B) A transverse section at the level of the prospective second visceral pouch of the pharynx (p) in a stage 9 embryo, in which endoderm contacting the surface ectoderm expresses GDNF. (C) Dorsal view of a stage 13 embryo, showing nonspecific hybridization in the brain. Specific expression in the pharynx (p), floor plate (fp), and nephrogenic cord (ne) is indicated by arrows. (D) A transverse section at the level of the second visceral pouch. The endodermal lining of the pharynx (p) contacting the surface ectoderm (arrowheads) expresses GDNF. (E) Ventral view of a stage 18 embryo. Arrows: expression in the floor plate (fp), foregut (fg), nephrogenic cord (ne), hindgut (hg), and allantois (al). (F) Expression in the mesenchyme (arrowheads) around the primary head veins (hv: arrows). (G) The head region of the embryo in E. Placode-derived distal ganglia of the facial (VII) and glossopharyngeal (IX) nerves are indicated by arrows. Arrowheads: a discrete expression domain located dorsal to the optic cup (op). ot, otocyst; pa, pharyngeal arch. Expression in the sinus venosus (sv) is indicated by an arrow. (H) A frontal section through the pharyngeal arches (pa). Epithelial lining of the pharyngeal cleft expresses GDNF. (I) A transverse section in the rostral brachial region of a stage 18 embryo. Arrowheads indicate unilateral expression of GDNF in the mesodermal wall of the foregut and the arrow indicates expression in the floor plate (fp). nc, notochord; da, dorsal aorta; dm, dorsal mesentery. The asterisk in I indicates the lumen of the foregut. (J) A transverse section in the upper thoracic region of a stage 18 embryo. The arrow indicates expression of GDNF in the floor plate (fp). ne, nephrogenic mesenchyme. Scale bars are 400 μm in A, 600 μm in C, 200 μm in E and G, and 100 μm in B, D, F, and H–J.

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GDNF Expression

Stage 10. Two regions of focal expression of GDNF mRNA were found in the head of embryos: small, localized expression in the anterior head and a more extensive expression in the caudal head (Fig. 2A). In transverse sections through the caudal region, expression was observed in the lateral epithelial lining of the pharynx under the surface ectoderm (Fig. 2B). GDNF expression was also found in the pronephric intermediate mesoderm and in the intermediate mesoderm at the level of newly segmented somites along the rostro-caudal axis (Fig. 2A). At the earliest examined here (stages 9–10), GDNF was not expressed anywhere within the central nervous system (CNS). However, soon after neural tube closure (stage 11 and onward), weak expression of GDNF was observed in the floor plate at the most rostral region of the spinal cord.

Stage 12. The expression pattern of GDNF mRNA was similar to stage 10 (Fig. 2C). In transverse sections through levels posterior to the otocysts, GDNF was expressed in regions of the endodermal lining of the pharynx that contacted the placodal thickening (Fig. 2D). At stage 14, GDNF expression in the floor plate now extended from cervical to upper brachial levels.

Stage 18. In the craniofacial region, GDNF was expressed in the distal ganglia of the VIIth and IXth cranial nerves (Fig. 2G). GDNF mRNA was also observed at the base of the clefts between the branchial arches (Figs. 2E and 2G). In sections, this expression was observed to be in the

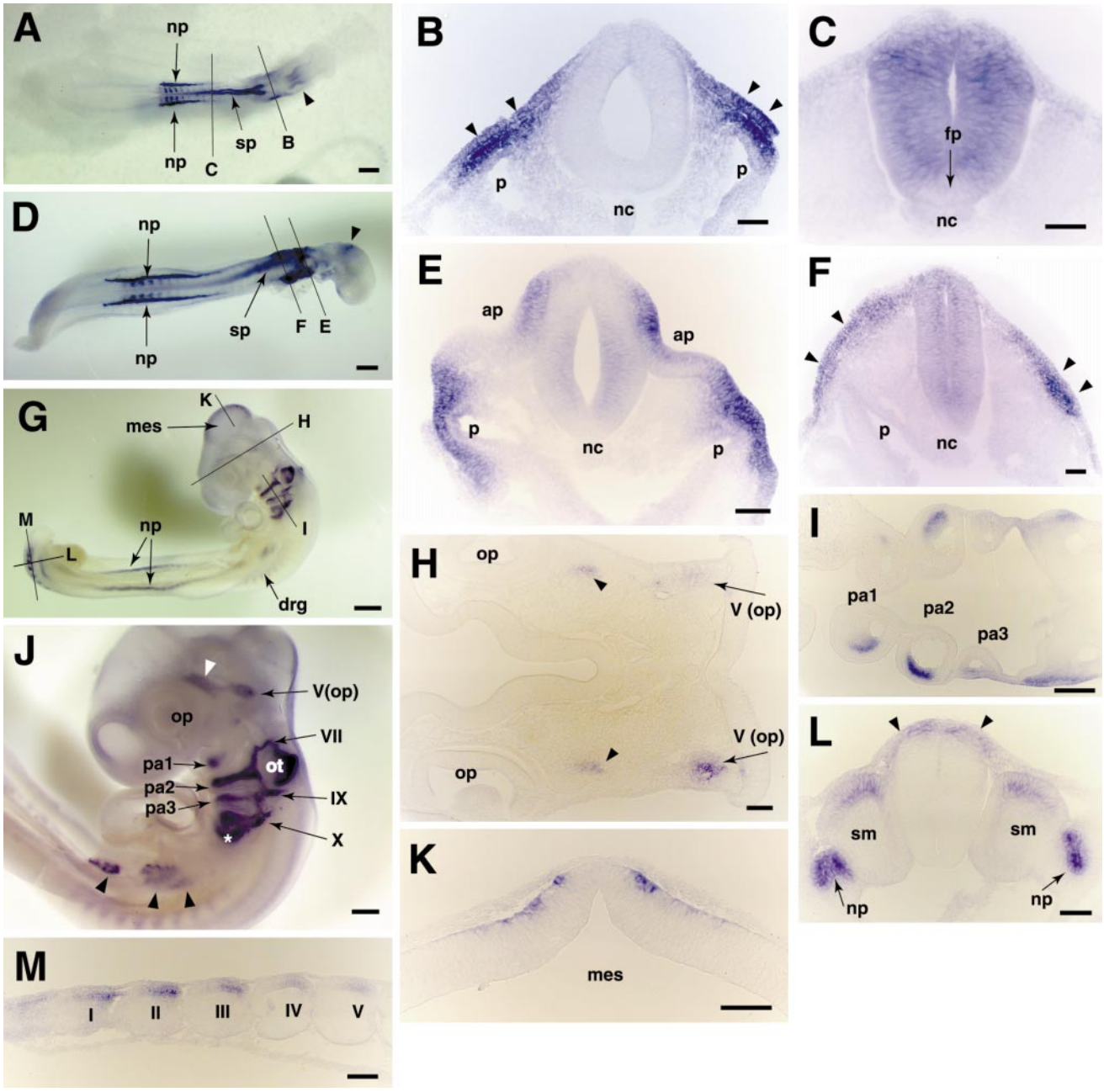


FIG. 3. The expression pattern of *c-ret* (RET). Whole-mount *in situ* hybridization (A, D, G, and J) and 30-μm sections (B, C, E, F, H, I, K, L, and M) at the approximate levels indicated by thin lines in A, D, and G. (A) Dorsal view of a stage 9 embryo. An arrowhead indicates hybridization in the mesen-rhombencephalic junction. Arrows: expression in the spinal cord (sp) and nephric duct (np). (B) A transverse section through the caudal rhombencephalon at stage 9. Arrowheads indicate hybridization in the surface ectoderm. RET is also expressed in the mesenchyme between the ectoderm and pharynx (p). nc, notochord. (C) A transverse section at the mid-level of the closed neural tube. The floor plate (fp) lacks the expression of RET. nc, notochord. (D) Dorsal view of a stage 13 embryo. The arrowhead indicates expression at the mesen-rhombencephalic junction. Arrows: expression in the spinal cord (sp) and nephric duct (np). (E) A transverse section through the auditory pit (ap) of a stage 13 embryo. Hybridization is observed in the dorsal half of the auditory placode as well as in the epibranchial placode, which contacts the endoderm of the pharynx (p). nc, notochord. (F) A transverse section through the level of upper cervical spinal cord from a stage 13 embryo. Arrowheads indicate RET expression in the surface ectoderm. p, pharynx; nc, notochord. (G) A stage 18 embryo, in which only the most caudal somites express RET. Hybridization in the dorsal root ganglia (drg) is indicated by an arrow. The nephric duct (np) and mesencephalon (mes), in which hybridization is observed in the dorsal midline, are also shown. (H) A transverse section through the optic cup (op) and ophthalmic division of the trigeminal ganglion (V (op), arrows) from a stage 18 embryo.

epithelial lining of the pharyngeal clefts (Fig. 2H). A cluster of GDNF-expressing cells was observed dorsally to the optic cups (Fig. 2G). This cluster did not appear to be the ophthalmic lobe of the trigeminal ganglion, because in frontal sections through the clusters, mesenchyme located ventral to the primary head veins was the major tissue expressing GDNF (Fig. 2F).

GDNF transcripts were also found in the mesonephric and metanephric kidneys (Fig. 2E) and in sections, GDNF was in mesenchyme cells adjacent to the ureteric bud (Fig. 2J).

GDNF expression in the floor plate now extended into the lower brachial and thoracic spinal cord (Fig. 2E). At stage 20, GDNF expression in the floor plate occurred at all levels of the spinal cord and persisted until stage 22. However, by stage 24, GDNF expression was no longer detected at any level of the spinal cord (not shown). Thus, GDNF expression in the floor plate is transient and either precedes or occurs coincident with the genesis of motoneurons along the spinal cord (Hollyday and Hamburger, 1977).

GDNF expression occurred in the digestive tract, where strong GDNF expression was located in the prospective stomach segment of the foregut and the allantois, whereas only weak expression was observed along other segments of the foregut and hindgut (Fig. 2E). In sections through the prospective stomach, strong expression of GDNF was observed in the mesoderm layer on the left side and a diffuse expression occurred in the entire mesenchyme surrounding the lumen of the foregut (Fig. 2I). In sections of the allantois, GDNF expression was observed in the epithelial lining of the lumen of the allantois (not shown). A portion of the wall of the sinus venosus expressed GDNF mRNA (Fig. 2E and 2G).

RET Expression

Stage 10. In the CNS, the expression of the *c-ret* protooncogene (RET) was observed at the mesen-rhombencephalic junction (Fig. 3A). The closed neural tube (but not the unclosed neural plate) abundantly expressed RET mRNA (Fig. 3A). In transverse sections of the neural tube, RET mRNA was observed in the entire neural tube except for the floor plate (Fig. 3C).

Outside of the central nervous system, RET mRNA was observed bilaterally, adjacent (but lateral) to the rhombencephalon (Fig. 3A). In sections thorough this region, RET was found in the mesenchyme beneath the surface ectoderm as well as in the surface ectoderm itself (Fig. 3B). Based on the location, the surface ectoderm expression represents epibranchial placodes although they were not yet distinctively thickened (Fig. 3B). The mesenchymal expression may be migrating neural crest cells, because emigration of neuronal precursors from the epibranchial placode generally begins later around stage 14 (D'Amico and Noden, 1983).

The expression of RET mRNA also occurred in the somites (Fig. 3A). However, the expression of RET was restricted to several newly segmented somites (somite developmental stages from I to IV or V; see Christ and Ordahl, 1995) and had disappeared from the older somites. In addition to the segmented somites, unsegmented paraxial mesoderm just caudal to the last segmented somites weakly expressed RET mRNA, and expression was also observed in the intermediate mesoderm (Fig. 3A: pronephron).

Stage 13. The expression of RET mRNA in the spinal cord exhibited a rostral to caudal pattern (Fig. 3D) and expression persisted at the mesen-rhombencephalic junction (Fig. 3D).

In the head and neck, RET expression was observed laterally at the level of the auditory pit and at the junction between the rhombencephalon and the cervical spinal cord (Fig. 3D). In transverse sections, RET expression occurred in thickened surface ectoderm (placode) and underlying mesenchyme, but not in the endodermal lining of the visceral pouches (Fig. 3E). In sections from more caudal regions, both the surface ectoderm and the underlying mesenchyme expressed RET mRNA laterally (Fig. 3F). Based on the location and morphology, these RET-expressing tissues represent epibranchial placodes that give rise to the distal ganglia of the facial, glossopharyngeal, and vagus nerves and delaminated cells derived from the placodes. Invaginating otocysts also expressed RET mRNA and in sections, RET expression was found in the dorsal half of the auditory placode (Fig. 3E).

Arrowheads indicate hybridization in the mesenchyme located dorsal to the optic cup. (I) A frontal section through the pharyngeal arches (pa) of a stage 18 embryo. Hybridization is clearly localized to the inner portion of the 1st pharyngeal arch. (J) The head region from a stage 18 embryo. Ophthalmic division of the trigeminal ganglion, V(op), distal ganglia of the facial (VII), glossopharyngeal (IX), and vagus (X) nerves are indicated by arrows. The white arrowhead in the dorsal region of the optic cup (op) indicates hybridization in a cluster of mesenchyme cells that is separated from the trigeminal ganglion. The white asterisk indicates expression in the surface ectoderm posterior to the fourth pharyngeal arch. Arrowheads indicate expression in migrating neural crest cells in the foregut. ot, otocyst; pa, pharyngeal arch. (K) A transverse section of the dorsal region of the mesencephalon (mes) from a stage 18 embryo. (L) A transverse section of a somite (sm) at somite developmental stage II. Hybridization in the somite is restricted to the dorsomedial region. Arrowheads indicate expression in the neural crest. The nephric duct (np) also expresses RET (arrows). (M) A parasagittal section of newly formed somites in a stage 18 embryo. Roman numerals indicate the developmental stage of somites as defined by Christ and Ordahl (1995). Scale bars: 400 μm in A and G; 200 μm in D and J; and 100 μm in B, C, E, F, H, I, K, L, and M.

RET expression was still restricted to newly formed somites (Fig. 3D) and the mesonephric duct now expressed RET mRNA (Fig. 3D).

Stage 18. In the CNS, RET mRNA expression occurred in several large cells located along the dorsal midline of the mesencephalon (Figs. 3G and 3K). These were the only cells expressing RET within the CNS since the earlier expression in the spinal cord had now disappeared. In the peripheral nervous system (PNS), the sensory ganglia of the ophthalmic division of the trigeminal (Vth) as well as distal ganglia of the facial (VIIth: geniculate ganglia), glossopharyngeal (IXth: petrosal ganglia), and vagus (Xth: nodose ganglia) nerves all expressed RET mRNA. The epibranchial placodes giving rise to the petrosal and nodose ganglia continued to express RET mRNA (Fig. 3J). Premigratory neural crest in the lumber spinal cord, but not in other spinal segments, expressed RET mRNA (Fig. 3L). In the cervical and upper brachial levels, aggregating dorsal root ganglion cells first began to express RET mRNA (Fig. 3G). Some cell populations in the mesenchymal layer of the foregut expressed RET mRNA (Fig. 3J). Neural crest cells that differentiate into myenteric neurons invade the wall of the foregut on Embryonic Day 3 (Tucker *et al.*, 1986) and therefore RET-expressing cells may be neural crest-derived immature myenteric ganglion cells. However, neither GFRα-1 nor GFRα-2 was expressed in these cell populations at these stages.

In the head region, clusters of RET mRNA expression were observed in the dorsal region of the optic cup (Fig. 3J). The expression of RET in these clusters occurred in mesenchyme cells located relatively deep within the embryo; they could be distinguished from the ophthalmic lobe of the trigeminal ganglia and appeared to be the same cell population that expressed GDNF mRNA (Figs. 3H and 3J, also see Figs. 2G and 2F). The dorsal half of the otocyst expressed RET mRNA, similar to earlier stages (Fig. 3J). Expression also occurred in the first, second, third, and fourth branchial arches (Figs. 3G and 3J). In frontal sections of the branchial arches, RET-expressing cells were located in the central portion of the arches and were separated from the surface ectoderm and surrounded by nonexpressing cells (Fig. 3I). The surface ectoderm and underlying mesenchyme cells located just posterior to the fourth branchial arch also expressed RET and these same regions also expressed GFRα-1 and 2 mRNA (see below). However, the identity of the cells in this region is unclear.

Expression in the somites was similar to that observed at earlier stages (Fig. 3G). In sagittal sections of the somites, the anterior-dorsal quadrants selectively expressed RET (Fig. 3M).

GFRα-1 Expression

Stage 10. In the CNS, the rostral mesencephalon adjacent to the prosencephalon expressed GFRα-1 mRNA (Fig. 4A). In transverse sections at this level, GFRα-1 expression was observed in the ventral region of the mesencephalon (Fig. 4B). GFRα-1 expression was absent from all other regions of the CNS at this stage.

Outside of the CNS, GFRα-1 mRNA expression occurred in Hensen's node, segmented somites, and mesenchyme flanking the midbrain and rostral hindbrain (Figs. 4A and 4C). GFRα-1 mRNA was also localized in the dorsal regions of the somite in transverse sections at somite developmental stage I (Fig. 4D). Pronephric intermediate mesoderm also expressed GFRα-1 mRNA (Figs. 4A and 4D).

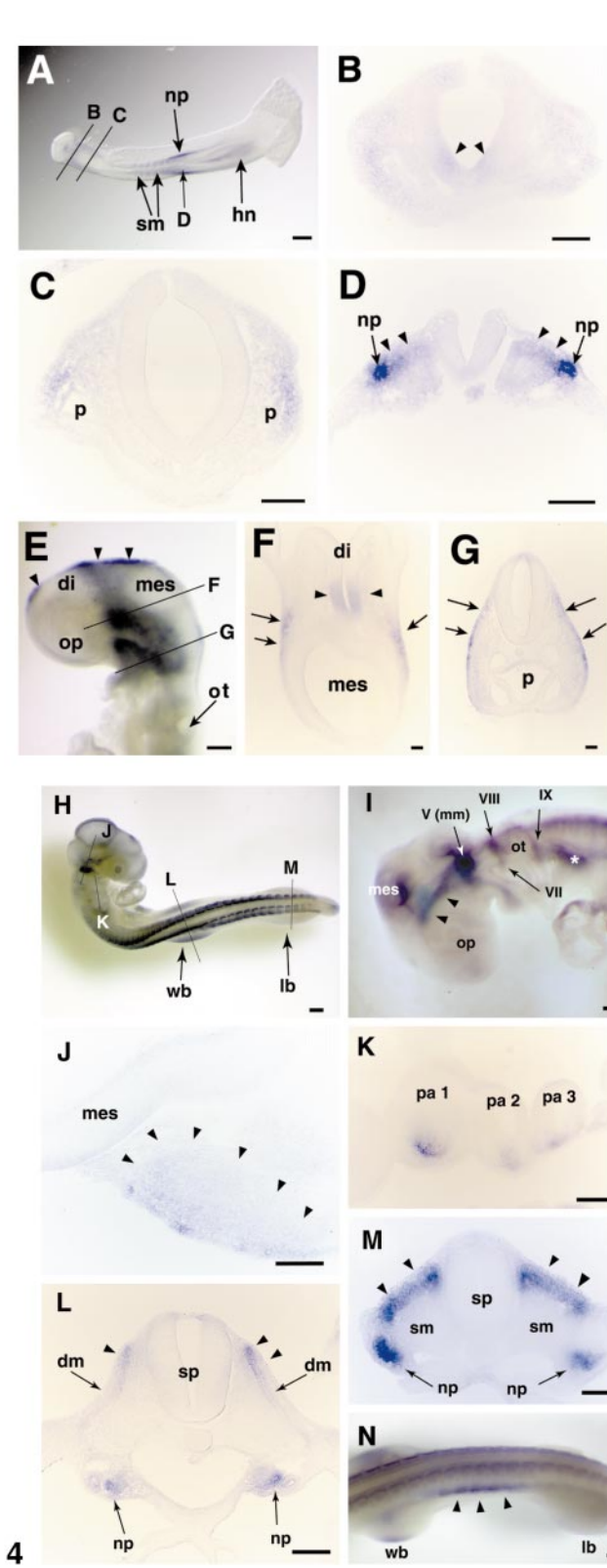
Stage 14. In the CNS, the dorsal midline of the diencephalon and rostral mesencephalon now expressed GFRα-1 mRNA (Fig. 4E) and the ventral neuroepithelium of the mesen-diencephalic junction also now expressed GFRα-1 (Figs. 4E and 4F).

Outside of the CNS, GFRα-1 mRNA was expressed in the surface ectoderm and underlying mesenchyme at the level of the mesencephalon and rostral rhombencephalon (Figs. 4E–4G) and the pattern of expression indicates that the ectodermal and some of the underlying mesenchymal expression of GFRα-1 may be in the trigeminal placode and delaminated ganglionic precursor cells derived from the placode.

Stage 18. Expression along the dorsal midline of the CNS was diminished at this stage except in the mesencephalon (Figs. 4H and 4I). In sections of the mesencephalon, expression was observed in the midline neuroepithelium, which was distinct from the region of RET expression. GFRα-1 expression also occurred in the marginal zone and in the ventral portion of the rostral mesencephalon (Figs. 4I and 4J).

In the head and neck regions, the sensory ganglia of the maxillo-mandibular division of the trigeminal (Vth), and the distal ganglia of the facial (VIIth), vestibuloacoustic (VIIIth), and glossopharyngeal (IXth) nerves expressed GFRα-1 mRNA (Figs. 4I and 4J). Within the maxillo-mandibular division of the trigeminal ganglia (Vth), expression was relatively concentrated in the lateral region of the ganglia in frontal sections (Fig. 4J). The expression of GFRα-1 also occurred in the mesenchyme surrounding primary head veins and in overlying ectoderm (Figs. 4I and 4J). Based on their location, GFRα-1-expressing cells in the ectoderm may represent the trigeminal placode and cells in the mesenchyme may be emigrating neuronal precursors derived from the placode. Internal mesenchyme in pharyngeal arches 1, 2, and 3 also expressed GFRα-1 (Fig. 4K). The expression pattern of cells in the pharyngeal arches appeared similar to that of RET. Discrete GFRα-1 expression domains in the ectoderm and underlying mesenchyme were observed in regions just caudal to the fourth pharyngeal arches (Fig. 4I).

The expression pattern of GFRα-1 in the somites was altered after stage 17. At stage 17, the dorsal lip of the dermamyotome expressed GFRα-1 mRNA at the cervical level, whereas the entire dorsal epithelium of the somite expressed GFRα-1 at more caudal levels (Figs. 4H and 4M). By stages 18–19, the dorsal lip of the dermamyotome expressed GFRα-1 at the cervical, brachial and lumber



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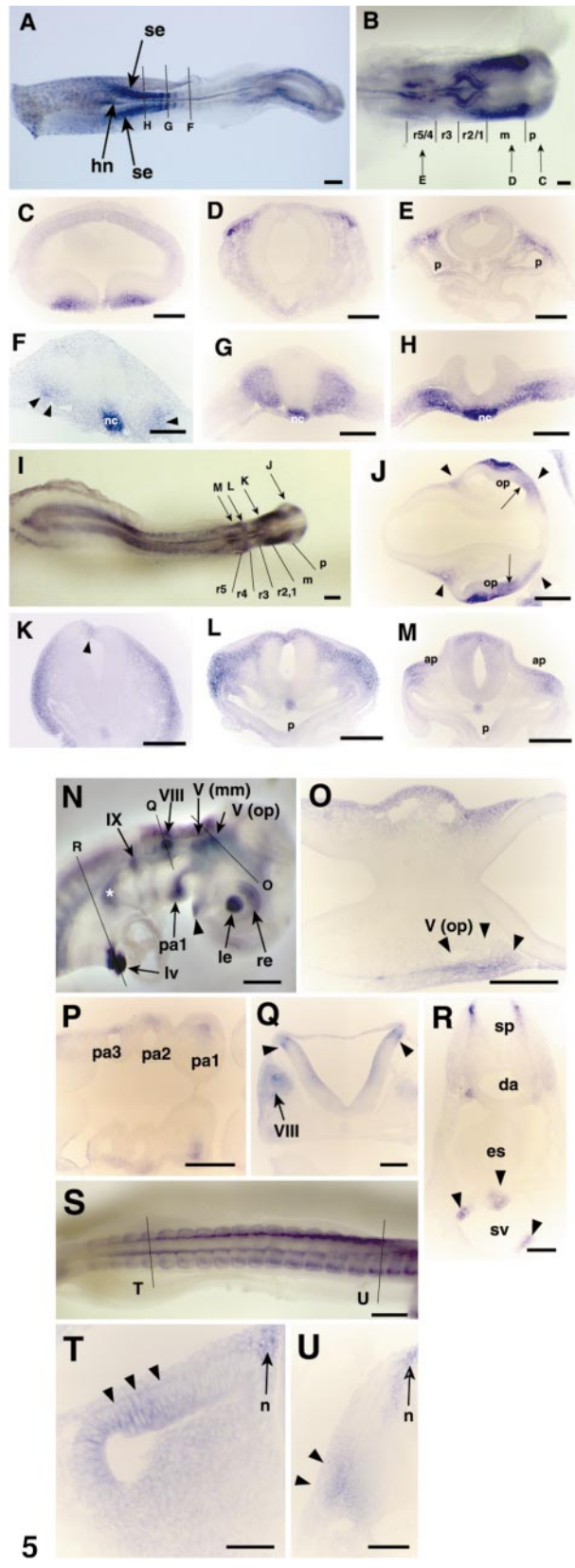

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levels, whereas the entire dermamyotome now expressed GFRα-1 only at the thoracic level (Figs. 4L and 4N).

Finally, the ureteric duct expressed GFRα-1 at this stage (Figs. 4L and 4M).

GFRα-2 Expression

Stage 10. In the CNS, the dorsal midline (neural crest) of rhombomeres 1/2, 3, and 4/5 all expressed GFRα-2

FIG. 4. Expression of GFRα-1. *In situ* analysis in whole-mount embryos (A, E, H, I, and N) or in 30-μm sections (B–D, F, G, and J–M). The level and orientation of the sections are shown in A, E, and H by thin lines or an arrow. (A) Dorsal view of an embryo at stage 9. The rostral mesencephalon and mesenchyme flanking the mesencephalon and rhombencephalon express GFRα-1. Arrows indicate hybridization in somites (sm), nephric duct (np), and Hensen's node (hn). (B) A transverse section through the junction between the prosencephalon and mesencephalon from a stage 9 embryo. Arrowheads indicate GFRα-1 expression in ventral neuroepithelial cells. (C) A transverse section through the middle of the mesencephalon from a stage 9 embryo. p, pharynx. Mesenchymal expression of GFRα-1 is observed. (D) A transverse section of the most caudal segmented somites from a stage 9 embryo. Arrowheads indicate the expression of GFRα-1 in the dorsal portion of the somite and arrows indicate expression in the nephric duct (np). (E) Lateral view of head from a stage 14 embryo. Arrowheads: hybridization along the dorsal midline in the CNS. di, diencephalon; mes, mesencephalon; op, optic cup; ot, otocyst. Hybridization is observed in the trigeminal placode (see F and G). (F) A horizontal section through the optic cup from a stage 14 embryo. Arrows indicate expression in the surface ectoderm (trigeminal placode) and underlying mesenchyme. Arrowheads indicate hybridization in the ventral part of the dien (di)-mesencephalic (mes) junction. (G) A transverse section at the approximate level of rhombomere 1–2 from a stage 14 embryo. Arrows: expression in the trigeminal placode and underlying mesenchyme. p, pharynx. (H) Dorsal view of trunk of a stage 17 embryo. GFRα-1 expression in the wing bud (wb) but not in the leg bud (lb). Hybridization is also observed in the somites and in the head. (I) Lateral view of the craniofacial region of a stage 18 embryo. Expression (arrows) is seen in the maxillo-mandibular division of the trigeminal ganglion, V (mm), vestibuloacoustic ganglion (VIII), and distal ganglia of the facial (VII) and glossopharyngeal (IX) nerves. Arrowheads indicate expression in the mesenchyme around the primary head vein as well as in the trigeminal placode. The white asterisk indicates hybridization in the ectoderm posterior to the 4th pharyngeal arch. mes, mesencephalon; op, optic cup; ot, otocyst. (J) A frontal section through the trigeminal ganglion of a stage 18 embryo. Arrowheads indicate the medial border of the ganglion. Hybridization is also observed in the placode adjacent to the ganglion. mes, mesencephalon. (K) A frontal section through pharyngeal arches (pa) 1, 2, and 3 from a stage 18 embryo. (L) A transverse section through the middle of the brachial spinal cord (sp) from a stage 18 embryo. Arrowheads indicate expression in the dorsal lip of the dermamyotome (dm, arrows). np, nephric duct. (M) A transverse section through a somite at somite developmental stage III at the leg bud level from a stage 18 embryo. Arrowheads indicate expression in the dorsal portion of the somite (sm). np, nephric duct (arrows); sp, spinal cord. (N) Dorsolateral view of the trunk from a stage 19 embryo. Arrowheads indicate expression in the ventral lip of the dermamyotome at the thoracic level between the wing bud (wb) and leg bud (lb). Scale bars are 400 μm for A and H; 100 μm for B–G, K, and M; and 200 μm for I, J, L, and N.

FIG. 5. GFRα-2 expression. *In situ* hybridization analysis in whole-mount embryos (A, B, I, N, and S) or 30-μm sections (C–H, J–M, O–R, T, and U). Thin lines or thin arrows with large capital letters in A, B, I, N, and S indicate the orientation and approximate level of the sections. (A) Dorsal view of an embryo at stage 9. Hybridization in the segmental plate (se) and Hensen's node (hn) are indicated by thick arrows. (B) Dorsal view of the head from an embryo at stage 9. Approximate boundaries of segmentation in the CNS are indicated by thin lines. p, prosencephalon; m, mesencephalon; r 2/1, rhombomere 1/2; r 3, rhombomere 3; r 5/4, rhombomere 4/5. (C, D, and E) Transverse sections through the prosencephalon, the middle of the mesencephalon, and the middle of rhombomere 4/5, respectively, from a stage 9 embryo. (F, G, and H) Transverse sections from a stage 9 embryo, showing expression patterns in the somite at somite developmental stage V in F, at stage I in G, and in the segmental plate in H. Expression is also observed in the notochord (nc). Arrowheads in F indicate expression in the ventrolateral portion of the somite. (I) Dorsal view of a stage 12 embryo. Segmental boundaries of prosencephalon (p), mesencephalon (m), and rhombomeres (r) 1–5 are indicated at the bottom of the figure. Levels of transverse sections in J–M are indicated at the top of the figure. (J–M) A transverse section of the prosencephalon in J, rhombomere 1 in K, rhombomere 4 in L, and rhombomere 5 in M from an embryo at stage 12. Arrowheads and arrows in J indicate mesenchymal expression around the optic cup (op) and hybridization in the ventral portion of the optic cup, respectively. Arrowheads in K indicate expression in the dorsal midline of rhombomere 1. The surface ectoderm (trigeminal placode) also expresses GFRα-2 in K. p, pharynx; ap, auditory pit. (N) Lateral view of the head and neck region from a stage 18 embryo. Arrows indicate expression in the dorsal retina (re), lens (le), ophthalmic (op) and maxillo-mandibular (mm) divisions of the trigeminal ganglion (V), vestibuloacoustic ganglion (VIII), distal ganglion of the glossopharyngeal nerve (IX), pharyngeal arch (pa), and liver primordium (lv). The white asterisk indicates ectodermal expression posterior to the 4th pharyngeal arch. The arrowhead indicates expression in the maxillary process. (O) A section through the plane indicated in N from a stage 18 embryo. Because this particular section is cut horizontally oblique, the ophthalmic lobe (op) of the trigeminal ganglion (V), indicated by arrowheads, is located in the lower part and mesenchymal expression around the head veins is seen in the upper part of the section. (P) A frontal section through pharyngeal arches (pa) 1–3 of a stage 18 embryo. (Q) A transverse section through vestibuloacoustic ganglion (VIII) from a stage 18 embryo. Arrowheads indicate hybridization in the dorsolateral region of the rhombencephalon. (R) A transverse section through the cervical level of an embryo at stage 18. Arrowheads indicate expression in the liver primordium. sp, spinal cord; da, dorsal aorta; sv, sinus venosus; es, esophagus. (S) Dorsolateral view of the trunk from a stage 17 embryo. (T) A transverse section of a somite at the level of the lower thoracic spinal cord from a stage 17 embryo. Arrowheads indicate expression in the ventrolateral portion of the somite. n, migrating neural crest cells. (U) A transverse section of the dermamyotome in the upper brachial region of a stage 17 embryo. Arrowheads indicate expression in the ventral lip of the dermamyotome and adjacent mesenchyme cells. n, migrating neural crest cells. Scale bars: 600 μm in A; 200 μm in B–E, I, N, S, and T; 100 μm in F–H, J–M, and O–R; 50 μm in U.

mRNA, whereas only the neuroepithelium of rhombomere 1/2 expressed GFRα-2 (Fig. 5B).

Head mesenchyme expressed GFRα-2 mRNA at all levels from the prosencephalon to the first somite (Figs. 5A and 5B). In cross sections, GFRα-2 mRNA was observed in mesenchyme cells located underneath the prosencephalon (Fig. 5C); in mesenchyme flanking the mesencephalon, with strong expression in cells beneath the dorsal ectoderm (Fig. 5D); and in mesenchyme cells located between the surface ectoderm and the endothelial lining of the pharynx at rhombencephalic levels (Fig. 5E). GFRα-2 expression was not observed in the surface ectoderm of these regions.

GFRα-2 expression occurred in segmented somites as well as in the unsegmented paraxial mesoderm (segmental plate; Fig. 5A). In transverse sections of the paraxial mesoderm, GFRα-2 mRNA was observed in the entire segmental plate and somites at stage I of somite development (Figs. 5G and 5H). In older somites (somite developmental stage III), expression in the medial portion close to the neural tube was diminished and was now relatively concentrated in the lateral portion of the somites. In somites at more rostral regions, GFRα-2 expression was restricted to the ventrolateral portion of the somite (Fig. 5F). Occasionally, GFRα-2 expression was observed in the floor plate and neural crest (Fig. 5F); however, this expression was not consistently seen in all embryos examined. Finally, GFRα-2 expression occurred in the notochord and Hensen's node (Figs. 5A and 5F–5H).

Stage 12. In the CNS, GFRα-2 expression was seen in the most dorsal region (neural crest) of rhombomeres 1, 2, 3, and 5 (Figs. 5I and 5K), whereas expression in the neural crest of rhombomere 4 had disappeared and, instead, a stream of GFRα-2-expressing cells emigrating from rhombomere 4 was now observed anterior to the invaginating auditory pit (Figs. 5I and 5L). Expression was found in the lens placode, in the ventral side of the evaginating optic cup (Fig. 5J), and on the ventral side of the invaginating otocyst (Fig. 5M). The dorsolateral surface ectoderm (placode for the trigeminal ganglia) also expressed GFRα-2 (Fig. 5K).

GFRα-2 hybridization in the head mesenchyme was segregated into three populations defined by location and by the amount of signal expression. The first population is in the mesenchyme surrounding the optic cup and exhibits modest expression levels (Figs. 5I and 5J). The second population comprises mesenchyme cells at the level of the mesencephalon and rhombomeres 1 and 2, which exhibit high expression levels (Figs. 5I and 5K). The last population is the mesenchyme located at the caudal rhombencephalic level showing a low level of expression (Figs. 5I and 5M). Mesenchymal expression of GFRα-2 was interrupted at the level of rhombomere 4 by cells streaming away from the rhombomere. At all levels, GFRα-2 expression was relatively concentrated in the lateral regions of the embryo (Figs. 5J–5M).

Expression patterns in the axial and paraxial mesoderm remained the same as at stage 10 (Fig. 5I).

Stage 18. Except for neuroepithelial cells located in the rhombencephalon, GFRα-2 expression was not seen in the CNS (Fig. 5Q). The positive neuroepithelial cells were composed of a continuous population of cells in rhombomeres 1 to 5, appearing as a thin line of expression in whole-mount preparations (Fig. 5N). The dorsal region of the optic cup and lens expressed GFRα-2 mRNA (Fig. 5N).

In the PNS, the trigeminal ganglion, including ophthalmic and maxillo-mandibular divisions, the vestibuloacoustic ganglia, and distal ganglia of the ninth nerve all expressed GFRα-2 (Figs. 5N, 5O, and 5Q). In the spinal cord, migrating neural crest cells expressed GFRα-2 mRNA (Figs. 5S–5U).

Mesenchymal expression of GFRα-2 was observed around the trigeminal ganglion, in the dorsal region of the optic cups and in maxillary processes (Figs. 5N and 5O). In sections, GFRα-2 hybridization in the mesenchyme around the trigeminal ganglion and optic cup was concentrated beneath the surface ectoderm (Fig. 5O). Because the overlying surface ectoderm also expressed GFRα-2, some of the mesenchymal expression around the trigeminal ganglion and optic cup may represent migrating neuronal precursors from the trigeminal placode. Mesenchyme cells in the inner core of arches 1, 2, 3, and 4 expressed GFRα-2 mRNA (Fig. 5P) and these appeared to be the same population that expresses GFRα-1 and RET. The surface ectoderm located posterior to the fourth arch expressed GFRα-2 mRNA (Fig. 5N) and this is also the same region that expresses GFRα-1 and RET.

In rostral regions of the trunk (cervical/brachial), GFRα-2 expression occurred in the ventral lip of the dermamyotome as well as in cells adjacent to the medial side of the ventral lip of the somite, which are probably migrating muscle precursor cells (Figs. 5S and 5U). At younger somite developmental stages, the ventrolateral portion of the somites also expressed GFRα-2 (Fig. 5T).

Finally, GFRα-2 expression was seen in the liver primordium descending through the wall of the sinus venosus (Figs. 5N and 5R).

GFRα-4 Expression

Stage 10. GFRα-4 mRNA was predominantly expressed in the axial and paraxial mesoderm and was observed in segmented somites, Hensen's node, and notochord (Fig. 6A). In transverse sections, expression was observed in the entire somite (Fig. 6B).

Stage 14. Somites continued to express GFRα-4 (Figs. 6C and 6E). In transverse sections from the cervical level, GFRα-4-expressing cells occurred in the marginal zone of the ventral spinal cord. Based on their location and timing of their differentiation, these cells are likely motoneurons. The dorsal half of the invaginating otocyst expressed GFRα-4 mRNA (Figs. 6C and 6D) and at caudal cervical and rostral brachial levels, the left side of the splanchnopleura expressed GFRα-4 mRNA (Figs. 6C and 6E) whereas at other levels, the splanchnopleura did not express GFRα-4.

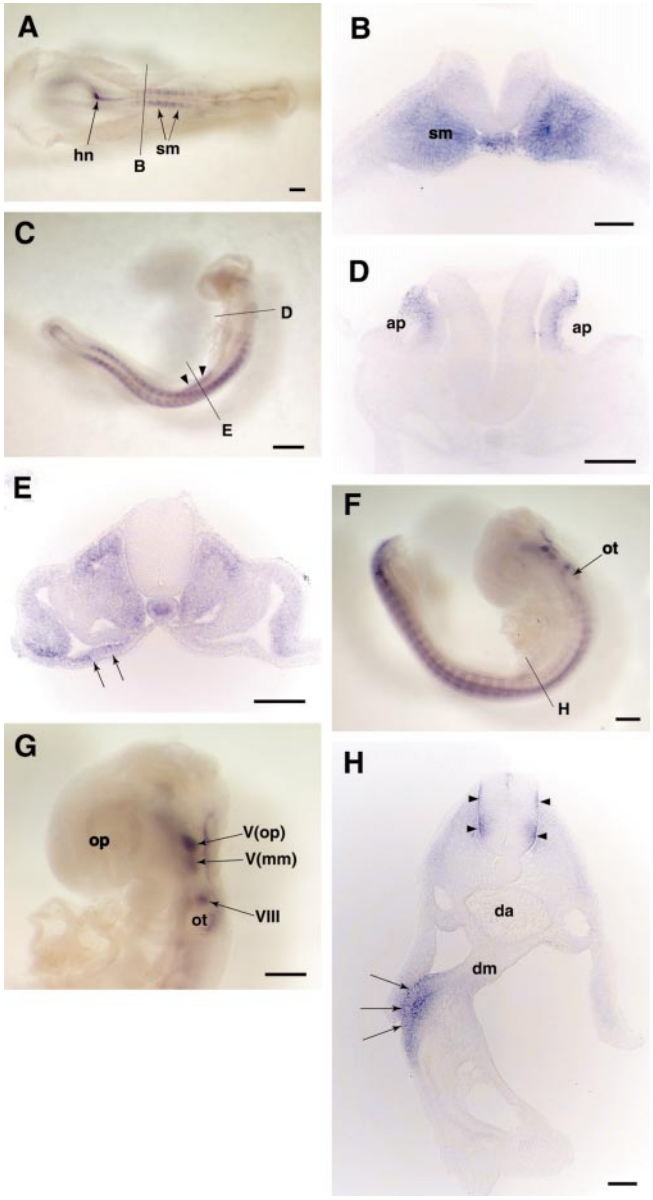

FIG. 6. Expression of GFRα-4. (A, C, F, and G) Whole-mount *in situ* hybridization in intact embryos. (B, D, E, and H) Expression in transverse sections. Approximate level of the sections is indicated in A, C, and F by thin lines. (A) Dorsal view of a stage 9 embryo. sm, somites; hn, Hensen's node. (B) A transverse section at the level of somite developmental stage I from a stage 9 embryo. sm, somites. (C) Lateral view of a stage 14 embryo. Arrowheads indicate expression in the left splanchnopleura. (D) A transverse section through the auditory pit (ap) from a stage 15 embryo. Expressions is localized in the dorsal half of the auditory placodes. (E) A transverse section at the approximate level indicated by the line in C. Arrows indicate expression in the left splanchnopleura. (F) Lateral view of a stage 18 embryo. ot, otocyst. (G) Lateral view of the head and neck regions from a stage 18 embryo. Expression in the ophthalmic (op) and maxillo-mandibular (mm) divisions of the trigeminal ganglion (V), and vestibuloacoustic ganglion (VIII) is indicated by arrows. op, optic cups; ot, otocyst. (H) A transverse section in the lower cervical region. Arrowheads indicate expression in cells located in the lateral region of the spinal cord, including motoneurons. Arrows indicate expression in the left splanchnopleura. da, dorsal aorta; dm, dorsal mesentery. Scale bars are 400 μm in A and C, 200 μm in F and G, and 100 μm in B, D, E, and H.

Stage 18. The expression pattern of GFRα-4 in the somites is similar to stage 14. However, in rostral somites, expression was diminished and only weak hybridization occurred around the ventral lip of the dermamyotome (Fig. 6F).

In the head and neck, three clusters of GFRα-4 mRNA-expressing cells were observed (Figs. 6F and 6G). Based on their location, these clusters represent the trigeminal ganglion (ophthalmic and maxillo-mandibular divisions) and the vestibuloacoustic ganglion. In addition, the mesenchyme surrounding these ganglia weakly expressed GFRα-4 mRNA and the dorsal region of the otocyst also expressed GFRα-4 (Figs. 6F and 6G).

In the hindbrain, the marginal zone of regions corresponding to rhombomere 1, 2, and 3 expressed GFRα-4 in a distinct area between dorsal and ventral neural tube and these cells were continuous along the rostro-caudal axis, appearing as a thin line in whole-mount preparations (Fig. 6G). In the spinal cord, cells located at the periphery of the dorsal neural tube as well as ventral motoneurons now exhibit expression at the brachial level (Fig. 6H).

The left side of the splanchnopleura expressed GFRα-4 mRNA at the level of the prospective stomach. This unilateral expression of GFRα-4 was restricted to the region of splanchnopleura ventral to the dorsal mesentery (Fig. 6H).

DISCUSSION

Expression during Somite Development

GFRα-1 and GFRα-2 are initially expressed in the dorsal and ventrolateral portion of newly developed somites, respectively. Subsequently, the expression of GFRα-1 and GFRα-2 become segregated in a complementary fashion such that GFRα-1 is restricted to the ventral lip of the dermamyotome, whereas GFRα-2 is in the dorsal lip of the dermamyotome. Because apaxial muscles form from the dorsomedial portion of the dermamytome, whereas hypaxial muscles are derived from the ventrolateral portion of the dermamyotome (Christ *et al.*, 1983; Ordahl and Le Douarin, 1992), GFRα-1 and GFRα-2 may represent molecular markers for these two muscle lineages.

Several molecular markers which differentially label dorsal or ventral subdomains of somites have been previously identified. Among them, *Pax-3* is expressed first in the segmental plate mesoderm, then in the dorsal half of stage I somites, and later in the dermamyotome especially in the ventral lip (Goulding *et al.*, 1994; Williams and Ordahl,

1994). Thus, although the initial expression patterns differ, the subsequent expression patterns of *Pax-3* and GFRα-2 are similar in the dermamyotome. Of the known myogenic regulatory factors (MRF), *myoD* is first expressed in the medial somite at somite stage II and later becomes restricted to the dorsomedial region of the somites in the avian embryo (Pownall and Emerson, 1992; Williams and Ordahl, 1994). Slightly later, *myf5* is expressed in the same region (Pownall and Emerson, 1992). GFRα-1 expression in the dorsal somite precedes the expression of *myoD* and is not restricted to the dorsomedial region of the dermamyotome until a relatively late stage of somite development. At the thoracic level, GFRα-1 is also expressed in the ventral lip of the dermamyotome. Another basic helix-loop-helix transcriptional factor, *Sim1,* which is a marker for lateral somites, is first expressed at stage 13 (Pourquié *et al.,* 1996). The somitic expression pattern of GFRαs differ from this and other known markers, suggesting that they might represent an independent and novel signaling pathway involved in myogenesis.

In the chick embryo, GDNF mRNA is expressed in the nephrogenic mesenchyme flanking newly differentiated somites. Later, GDNF expression occurs in the floor plate coincident with diminished expression in the intermediate mesoderm. Moreover, GDNF is only expressed in the floor plate in regions adjacent to segmented somites. The binding receptor component GFRα-1, which preferentially binds GDNF, is expressed in myogenic regions of the somite giving rise to apaxial muscles, whereas GFRα-2 is expressed in the hypaxial myogenic region of the somite. Although GFRα-2 preferentially binds another ligand, neurturin, GDNF can also bind to GFRα-2 under some conditions *in vitro* (Worby *et al.,* 1996; Baloh *et al.,* 1997; Sanicola *et al.,* 1997; Suvanto *et al.,* 1997). These results suggest that GDNF in the intermediate mesoderm and floor plate may influence the differentiation of apaxial and hypaxial muscles.

Many studies support the idea that a signal (Sonic hedgehog, Shh?) derived from ventral midline structures induces or promotes the differentiation of muscles cells and the expression of myogenic genes (Buffinger and Stockdale, 1994; Johnson *et al.,* 1994; Münsterberg and Lassar, 1995; Münsterberg *et al.,* 1995; Stern and Hauschka, 1995; Pownall *et al.,* 1996; Dietrich *et al.,* 1997). However, myogenic genes are still expressed in Shh-deficient mice (Chiang *et al.,* 1996). Therefore, factors other than Shh may also contribute to the induction of myogenic regulatory genes. The expression of GFRαs in the somites and GDNF in nearby tissues suggests that GDNF may be one such factor. The relatively late onset of GDNF expression in the floor plate indicates that it could be involved in the maintenance of expression of the MRF. Signals from the neural tube (Bober *et al.,* 1994), especially the ventral neural tube and floor plate (Dietrich *et al.,* 1997), are necessary for the maintenance of *MyoD* expression.

The GDNF signal transduction receptor component, RET, is only expressed in newly developing somites regardless of developmental age and thus is not associated with the expression patterns of GFRα-1 and 2, suggesting that GFRα-1 and 2 may utilize other signal transduction components. Conversely, RET may be involved in other developmental process such as segmentation of somites and may function independent of binding receptor components.

Expression during Craniofacial Development

The receptor components, RET, GFRα-1, and 2 are expressed in mesenchyme cells located in the medial regions of the branchial arches, whereas GDNF is expressed in the epithelial lining of the pharyngeal clefts. This close apposition of GDNF and the receptor components necessary for signal transduction indicates that GDNF from the epithelial lining could interact with RET and GFRα-1 (and possibly GFRα-2) in the branchial arches and thus may be involved in the development of pharyngeal arches. However, no gross facial abnormalities have been described in RET, GFRα-1 and 2, or GDNF-deficit mice (Schuchardt *et al.,* 1994; Moore *et al.,* 1996; Pichel *et al.,* 1996; Sánchez *et al.,* 1996; Cacalano *et al.,* 1998; Enomoto *et al.,* 1998; Rossi *et al.,* 1999). Although RET is not expressed in the branchial arches of mice (Pachnis *et al.,* 1993), the expression pattern of GDNF in chick and rodent is localized to the epithelial lining of the pharyngeal pouches (Sánchez *et al.,* 1996; Suvanto *et al.,* 1996). Thus, the biological action of GDNF in pharyngeal arch development may utilize different signal transduction components in chick and rodent.

In the head region at stages 17–18, the binding receptor components, GFRα-1 and 2, have a relatively broad and diffuse expression pattern in the mesenchyme cells around the trigeminal ganglion and optic cup, whereas RET and GDNF are expressed in discrete domains around the optic cup and appear to partly overlap with the GFRα-expressing cells. The identity of the mesenchyme cells expressing GFRα receptors is unclear. Because some of the mesenchyme cells appear to be associated with trigeminal placode, they are likely to be ganglionic precursors delaminated from the placode (see below). However, not all of the GFRα-positive mesenchyme cells are placode-derived. For example, the distribution and numbers of mesenchyme cells expressing GFRα-2 differ from those of migrating placode-derived neuronal precursors and are partly overlapping with migrating neural crest (Covell and Noden, 1989; Moody *et al.,* 1989). Head mesenchyme is composed principally of ectoderm-derived neural crest cells and mesoderm-derived paraxial mesoderm cells (Noden, 1986; Couly *et al.,* 1993; Noden, 1988), and these two subpopulations are extensively mixed during early developmental stages (Trainor and Tam, 1995). In the absence of specific markers, it is not possible to determine whether GDNF/receptor-positive cells are neural crest-derived that will give rise to connective tissues and cranial bones or mesoderm-derived cells that will give rise to muscle or placode-derived neuronal precursors. The local expression of RET could define sites of condensation of neural crest cells or meso-

TABLE 1
Expression of RET and GFRαs in Placodes and in Placode-Derived Ganglia

		Ganglionic expression			Placodal expression					
		RET	GFRα-1	GFRα-2	RET		GFRα-1		GFRα-2	
		St. 18	St. 18	St. 18	St. 10	St. 14	St. 10	St. 14	St. 10	St. 14
Trigeminal ganglia (V) (lateral part)	Ophthalomic division	+	−	+	−	−	−	+	−	+
	Maxillo-mandibular division	−	+	+						
Vestibuloacoustic ganglia (VIII)		−	+	+	−	+	−	−	−	+
Distal ganglia of the facial nerve (VII)		+	+	−	+	+	−	−	−	−
Distal ganglia of the glossopharyngeal nerve (IX)		+	+	+	+	+	−	−	−	−
Distal ganglia of the vagus nerve (X)		+	−	−	+	+	−	−	−	−

Note. The expression of RET and GFRα-2 is localized in the dorsal and ventral halves of the otocystes, respectively. St., stage.

derm cells to form bone or muscle, and the coexpression of GDNF with RET-expressing cells suggests possible autocrine or paracrine mechanisms of action.

Expression in the Peripheral and Central Nervous System

In addition to GDNF receptor expression in head mesenchyme, these receptors are also expressed in placodes as well as immature placode-derived ganglia (Table 1).

Avian neurogenic placodes are classified into three groups according to their position along the dorso-ventral axis: dorsolateral placodes give rise to the vestibuloacoustic ganglia; placodes for the trigeminal ganglia are located at intermediate levels; and epibranchial placodes located immediately dorsal to the branchial cleft give rise to distal ganglia of the VIIth, IXth, and Xth nerves (Ariens-Kappers, 1941; D'Amico-Martel and Noden, 1983). In these different ganglia, neurogenesis (delamination from the placode) generally begins around stages 13–14 and persists until stages 22–24 (D'Amico-Martel and Noden, 1983). Receptor expression in placodes and immature ganglia is characterized by the early onset of RET followed by later expression of GFRαs. This pattern is clearly seen in the epibranchial placode and its derived distal ganglia: RET is expressed already in the prospective placodal region of ectoderm, whereas GFRαs are first expressed after the immature ganglia are formed. This late onset of GFRαs in the placodes and ganglia suggests that GFRα expression may be associated with neuronal differentiation, and the initial survival of immature ganglionic cells (Moody *et al.*, 1989). The early onset of RET expression in the placodal ectoderm suggests an involvement in specification and induction of the placode, especially the epibranchial placode. The specification or induction of the trigeminal and otic placode is influenced by signals from the CNS (Waddington, 1937; Stark *et al.*, 1997; Baker *et al.*, 1999). Bone morphogenetic protein 7 (BMP7) released from the pharyngeal endoderm induces the expression of a molecular marker for the distal ganglia in ectoderm taken from head regions of the chick embryo *in vitro* (Begbie *et al.*, 1999). RET is broadly expressed in the prospective ectodermal placode region and GDNF is focally expressed in the endoderm of the prospective pharyngeal pouches, which are in contact with the surface ectoderm. These expression patterns suggest that GDNF and RET may also be involved in the specification and induction of the epibranchial placode. Once immature distal ganglia (geniculate and petrosal) are formed, they express both GFRα-1 and RET as well as GDNF. In these ganglia, GDNF may mediate later developmental events via autocrine or paracrine loops.

In the trunk region, migrating neural crest cells in the ventromedial pathway express GFRα-2 and RET, but not GFRα-1. This suggests that neurturin, a preferential ligand for the GFRα-2/RET complex, may be expressed along the neural crest migration pathways and could influence the differentiation or survival of these cells. Alternatively, GDNF, which can also bind to RET/GFRα-2, albeit with lower efficiency, could influence the differentiation of neural crest cells from its location in the floor plate. GDNF has been shown to promote the generation of adrenergic neurons in cultured neural crest cells (Maxwell *et al.*, 1996).

In the central nervous system, GDNF is transiently expressed only in the floor plate of the spinal cord and was not found in any other region at the stages observed here. In a previous study on the expression pattern of GDNF in the

mouse embryo, GDNF was reported to be expressed in the dorsal neuroepithelium of the spinal cord, but not in floor plate (Hellmich *et al.*, 1995). However, subsequent studies failed to observe GDNF in the dorsal spinal cord or floor plate of rodent embryos (Suvanto *et al.*, 1996; Wright and Snider, 1996). These apparent discrepancies may reflect either species or methodological differences. For instance, in order to demonstrate the presence of GDNF in the chick embryo floor plate required a strong digestion of proteinase K during *in situ* hybridization.

The expression of GDNF in the floor plate was only observed in the spinal cord and the onset of expression occurred after differentiation of the floor plate but prior to the differentiation of neuronal populations such as spinal interneurons and motoneurons (Yaginuma *et al.*, 1990). In rostral regions of the CNS, Sonic hedgehog (Shh) derived from ventral midline structures regulates the differentiation of specific cell types within the CNS in conjunction with other growth factors (Dale *et al.*, 1997; Ye *et al.*, 1998). Thus, GDNF in the floor plate could interact with Shh in promoting the differentiation of various neuronal phenotypes. Alternatively, GDNF may promote the survival of ventral midline cell populations. We have observed apoptotic cell death soon after the onset of GDNF expression in the brachial spinal cord (Homma *et al.*, 1994).

Asymmetrical Expression of GFRα-4 and GDNF

Several molecules have been identified that are preferentially expressed on the right or left side of embryos prior to the onset of morphogenetic events that mediate the asymmetrical movement of particular organs (Levin *et al.*, 1995, 1997; Meno *et al.*, 1996; Hyatt and Yost, 1998). One such factor, the transcription factor *Pitx2* is expressed along one lateral (left) side of the gastrointestinal tract (Logan *et al.*, 1998; Piedra *et al.*, 1998; Yoshioka *et al.*, 1998). Misexpression of *Pitx2* on the right side results in a reversal of gut rotation (Logan *et al.*, 1998). However, *Pitx2* is expressed throughout the visceral and cardiac mesoderm and presumably plays a similar role in the development of all asymmetric organs, whereas GDNF and GFRα-4 are only expressed in the prospective stomach region of the gut and their asymmetric expression pattern occurs relatively late in development, compared to other asymmetrically expressed factors. This unique spatial-temporal expression pattern is consistent with a role for GDNF and GFRα-4 in the late occurrence of leftward rotation of the stomach.

In conclusion, a detailed examination of the expression of GDNF and its receptors in early stages of chick embryo development has revealed a number of unique patterns that suggest novel functions for GDNF ligands in early organogenesis. Perturbation studies involving inhibition and overexpression of GDNF and its receptors *in vivo* are currently in progress in an attempt to experimentally test some of the suggested roles of these molecules in early chick development. Finally, a paper describing GDNF and receptor expression at later ages (Embryonic Days 4–12) is in preparation.

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REFERENCES

Ariens-Kappers, J. (1941). Kopfplakoden bei Wirbeltieren. *Ergeb. Ant. Entw.* **33,** 370–412.

Baloh, R. H., Tansey, M. G., Golden, J. P., Creedon, D. J., Heuckeroth, R. O., Keck, C. L., Zimonjic, D. B., Popescu, N. C., Johnson, E. M., Jr., and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* **18,** 793–802.

Baloh, R. H., Gorodinsky, A., Golden, J. P., Tansey, M. G., Keck, C. L., Popescu, N. C., Johnson, E. M., Jr., and Milbrandt, J. (1998a). GFRα3 is an orphan member of the GDNF/neurturin/persephin receptor family. *Proc. Natl. Acad. Sci. USA* **95,** 5801–5806.

Baloh, R. H., Tansey, M. G., Lampe, P. A., Fahrner, T. J., Enomoto, H., Simburger, K. S., Leitner, M. L., Araki, T., Johnson, E. M., Jr., and Milbrandt, J. (1998b). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRα3-RET receptor complex. *Neuron* **21,** 1291–1302.

Baker, C. V. H., Stark, M. R., Marcelle, C., and Bronner-Fraser, M. (1999). Competence, specification and induction of Pax-3 in the trigeminal placode. *Development* **126,** 147–156.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A., and Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373,** 339–341.

Begbie, J., Brunet, J.-F., Rubenstein, J. L. R., and Graham, A. (1999). Induction of the epibranchial placodes. *Development* **126,** 895–902.

Bober, E., Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R., Paterson, B. M., Arnold, H. H., and Christ, B. (1994). Initial steps of myogenesis in somites are independent of influence from axial structures. *Development* **120,** 3073–3082.

Buffinger, N., and Stockdale, F. E. (1994). Myogenic specification in somites: induction by axial structures. *Development* **120,** 1443–1452.

Buj-Bello, A., Adu, J., Piñón, L. G. P., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V. L., and Davis, A. M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. *Nature* **387,** 721–724.

Cacalano, G., Fariñas, I., Wang, L.-C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A. M., Reichardt, L. F., Hynes, M., Davis, A., and Rosenthal, A. (1998). GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21,** 53–62.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383,** 407–413.

Choi-Lundberg, D. L., and Bohn, M. C. (1995). Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. *Dev. Brain Res.* **85,** 80–88.

Christ, B., Jacob, M., and Jacob, H. J. (1983). On the origin and development of the ventrolateral abdominal muscles in the avian embryos. An experimental and ultrastructual study. *Anat. Embryol.* **166,** 87–101.

Christ, B., and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat. Embryol.* **191,** 381–396.

Couly, G. F., Coltey, P. M., and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: A study in quail-chick chimeras. *Development* **117,** 409–429.

Covell, D. A., Jr., and Noden, D. M. (1989). Embryonic development of the chick primary trigeminal sensory-motor complex. *J. Comp. Neurol.* **286,** 488–503.

Creedon, D. J., Tansey, M. G., Baloh, R. H., Osborne, P. A., Lampe, P. A., Fahrner, T. J., Heuckeroth, R. O., Milbrandt, J., and Johnson, E. M., Jr. (1997). Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **94,** 7018–7023.

Cristina, N., Chatellard-Causse, C., Manier, M., and Feuerstein, C. (1995). GDNF: Existence of a second transcript in the brain. *Mol. Brain Res.* **32,** 354–357.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J., and Placzek, M. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90,** 257–269.

Davis, S., Aldrich, T. H., Ip, N. Y., Stahl, N., Scherer, S., Farruggella, T., DiStefano, P. S., Curtis, R., Panayotatos, N., Gascan, H., Chevalier, S., and Yancopoulos, G. D. (1993). Released form of CNTF receptor α component as a soluble mediator of CNTF responses. *Science* **259,** 1736–1739.

D'Amico-Martel, A., and Noden, D. M. (1983). Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. *Am. J. Anat.* **166,** 445–468.

Dietrich, S., Schubert, F. R., and Lumsden, A. (1997). Control of dorsoventral pattern in the chick paraxial mesoderm. *Development* **124,** 3895–3908.

Enokido, Y., de Sauvage, F., Hongo, J.-A., Ninkina, N., Rosenthal, A., Buchman, V. L., and Davis, A. M. (1998). GFRα-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. *Curr. Biol.* **8,** 1019–1022.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M., Jr., and Milbrandt, J. (1998). GFRα1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21,** 317–324.

Golden, J. P., Baloh, R. H., Kotzbauer, P. T., Lampe, P. A., Osborne, P. A., Milbrandt, J., and Johnson, E. M., Jr. (1998). Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. *J. Comp. Neurol.* **398,** 139–150.

Goulding, M., Lumsden, A., and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermamyotome and its role in muscle development. *Development* **120,** 957–971.

Grimm, L., Holinski-Feder, E., Teodoridis, J., Scheffer, B., Schindelhauer, D., Meitinger, T., and Ueffing, M. (1998). Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3′-UTR and alternative splice products. *Hum. Mol. Genet.* **12,** 1873–1886.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hellmich, H. L., Kos, L., Cho, E. S., Mahon, K. A., and Zimmer, A. (1996). Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech. Dev.* **54,** 95–105.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A., Koliatsos, V. E., and Rosenthal, A. (1994). GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266,** 1062–1064.

Hollyday, M., and Hamburger, V. (1977). An autoradiographic study of the formation of the lateral motor column in the chick embryo. *Brain Res.* **132,** 197–208.

Homma, S., Yaginuma, H., and Oppenheim, R. W. (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: A possible means of early phenotypic selection. *J. Comp. Neurol.* **345,** 377–395.

Hyatt, B. A., and Yost, H. Y. (1998). The left-right coordinator: The role of Vg1 in organizing left-right axis formation. *Cell* **93,** 37–46.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69,** 1121–1132.

Jing, S., Wen, D., Yu, Y., Holst, P., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-L., Hu, S., Altrock, B. W., and Fox, G. M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. *Cell* **85,** 1113–1124.

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P. L., Boone, T., Delaney, J., Schultz, H., Zhou, R., and Fox, G. M. (1997). GFRα-2 and GFRα-3 are two new receptors for ligands of the GDNF family. *J. Biol. Chem.* **272,** 33111–33117.

Johnson, R. L., Laufer, E., Riddle, R. D., and Tabin, C. (1994). Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. *Cell* **79,** 1165–1173.

Klein, R. D., Sherman, D., Ho, W.-H., Stone, D., Bennett, G. L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.-A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C. E., Takahashi, M., and Rosenthal, A. (1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. *Nature* **387,** 717–721.

Kotzbauer, P. T., Lampe, P. A., Heuckeroth, R. O., Golden, J. P., Greedon, D. J., Johnson, E. M., Jr., and Milbrandt, J. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384,** 467–470.

Lin, L.-F., Dohetry, D. H., Lile, J. D., Bektesh, S., Collins, F. (1993). GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260,** 1130–1132.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. R., and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82,** 803–814.

Levin, M., Pagan, S., Roberts, D. J., Cooke, J., Kuehn, M. R., and Tabin, C. J. (1997). Left/right patterning signals and the independent regulation of different aspects of situs in the chick embryo. *Dev. Biol.* **189,** 57–67.

Logan, M., Pagán-Westphal, S. M., Smith, D. M., Paganessi, L., and Tabin, C. J. (1998). The transcriptional factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94,** 307–317.

Luukko, K., Suvanto, P., Saarma, M., and Thesleff, I. (1997). Expression of GDNF and its receptors in developing tooth is developmentally regulated and suggests multiple roles in innervation and organogenesis. *Dev. Dynam.* **210,** 463–471.

Luukko, K., Saarma, M., and Thesleff, I. (1998). Neurturin mRNA expression suggests roles in trigeminal innervation of the first branchial arch and in tooth formation. *Dev. Dynam.* **213,** 207–219.

Maxwell, G. D., Reid, K., Elefanty, A., Bartlett, P. F., and Murphy, M. (1996). Glial cell line-derived neurotrophic factor promotes the development of adrenergic neurons in moue neural crest cultures. *Proc. Natl. Acad. Sci. USA* **93,** 13274–13279.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y., and Hamada, H. (1996). Left-right asymmetric expression of the TGFβ-family member lefty in mouse embryos. *Nature* **381,** 151–155.

Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S., Golden, J. P., Davies, J. A., Vejsada, R., Kato, A. C., Hynes, M., Sherman, D., Nishimura, M., Wang, L. C., Vandlen, R., Moffat, B., Klein, R. D., Poulsen, K., Gray, C., Garces, A., Henderson, E. H., Phillips, H. S., and Johnson, E. M., Jr. (1998). Persephin, a novel neurotrophic factor related to GDNF and Neurturin. *Neuron* **20,** 245–253.

Moody, S. A., Quigg, M. S., and Frankfurter, A. (1989). Development of the peripheral trigeminal system in the chick revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. *J. Comp. Neurol.* **279,** 567–580.

Moore, M. W., Klein, R. D., Fariñas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382,** 76–79.

Münsterberg, A. E., and Lassar, A. B. (1995). Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. *Development* **121,** 651–660.

Münsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P., and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9,** 2911–2922.

Naveilhan, P., Baudet, C., Mikaels, Å., Shen, L., Westphal, H., and Ernfors, P. (1998). Expression and regulation of GFRα3, a glial cell line-derived neurotrophic factor family receptor. *Proc. Natl. Acad. Sci. USA* **95,** 1295–1300.

Noden, D. M. (1986). Patterning of avian craniofacial muscles. *Dev. Biol.* **116,** 347–356.

Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103**(Suppl.), 121–140.

Nosrat, C. A., Tomac, A., Lindqvist, E., Lindoskog, S., Humpel, C., Strömberg, I., Ebendal, T., Hoffer, B. J., and Olson, L. (1996). Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. *Cell Tissue Res.* **286,** 191–207.

Nosrat, C. A., Tomac, A., Hoffer, B. J., and Olson, L. (1997). Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor-α mRNAs. *Exp. Brain Res.* **115,** 410–422.

Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, L.-F. H., Li, L., Lo, A. C., Newsome, A. L., Prevette, D. M., and Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* **373,** 344–346.

Ordahl, C. P., and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114,** 339–353.

Pachnis, V., Mankoo, B., and Costantini, F. (1993). Expression of the *c-ret* proto-oncogene during mouse embryogenesis. *Development* **119,** 1005–1017.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., and Westphal, H. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382,** 73–76.

Piedra, M. E., Icardo, J. M., Albajar, M., Rodriguez-Rey, J. C., and Ros, M. A. (1998). *Pitx2* participates in the late phase of the pathway controlling left-right asymmetry. *Cell* **94,** 319–324.

Pourquié, O., Fan, C.-M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-Wewst, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: A role for BMP4. *Cell* **84,** 461–471.

Pownall, M. E., and Emerson, C. P., Jr. (1992). Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. *Dev. Biol.* **151,** 67–79.

Pownall, M. E., Strunk, K. E., and Emerson, C. P., Jr. (1996). Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. *Development* **122,** 1475–1488.

Rossi, J., Luukko, K., Poteryaev, D., Laurikainen, A., Sun, Y. F., Laakso, T., Eerikäinen, S., Tuominen, R., Lasko, M., Rauvala, H., Arumäe, U., Pasternack, M., Saarma, M., and Airaksinen, M. S. (1999). Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. *Neuron* **22,** 243–252.

Sánchez, M. P., Silos-Santigo, I., Frisén, J., He, B., Lira, S. A., and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382,** 70–73.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R. B., and Cate, R. L. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. *Proc. Natl. Acad. Sci. USA* **94,** 6238–6243.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367,** 380–383.

Schuchardt, A., Srinivas, S., Pachnis, V., and Costantini, F. (1995). Isolation and characterization of a chick homolog of the c-*ret* proto-oncogene. *Oncogene* **10,** 641–649.

Springer, J. E., Seeburger, J. L., He, J., Gabrea, A., Blankenhorn, E. P., and Bergman, L. W. (1995). cDNA sequence and differential mRNA regulation of two forms of glial cell line-derived neurotrophic factor in Schwann cells and rat skeletal muscle. *Exp. Neurol.* **131,** 47–52.

Stark, M. R., Sechrist, J., Bronner-Fraser, M., and Marcelle, C. (1997). Neural tube-ectoderm interactions are required for trigeminal placode formation. *Development* **124,** 4287–4295.

Stern, H. M., and Hauschka, S. D. (1995). Neural tube and notochord promote *in vitro* myogenesis in single somite explants. *Dev. Biol.* **167,** 87–103.

Suter-Crazzolara, C., and Unsicker, K. (1994). GDNF is expressed in two forms in many tissues outside the CNS. *NeuroReport* **5,** 2486–2488.

Suvanto, P., Hiltunen, J. O., Arumäe, U., Moshnyakov, M., Sariola, H., Sainio, H., and Saarma, M. (1996). Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by *in situ* hybridization. *Eur. J. Neurosci.* **8,** 816–822.

Suvanto, P., Wartiovaara, K., Lindahl, M., Arumäe, U., Moshnyakov, M., Horelli-Kuitunen, N., Airaksinen, M. S., Palotie, A., Sariola, H., and Saarma, M. (1997). Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptorβ, a homologue to GDNFR-α. *Hum. Mol. Genet.* **6,** 1267–1273.

Thompson, J., Doxakis, E., Piñón, L. G. P., Strachan, P., Buj-Bello, A., Wyatt, S., Buchman, V. L., and Davis, A. M. (1998). GFRα-4, a new GDNF family receptor. *Mol. Cell. Neurosci.* **11,** 117–126.

Tomac, A., Lindqvist, E., Lin, L.-F. H., Ögren, S. O., Young, D., Hoffer, B. J., and Olson, L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* **373,** 335–339.

Trainor, P. A., and Tam, P. P. (1995). Cranial paraxial mesoderm and neural crest cells of the mouse embryo: Codistribution in the craniofacial mesenchyme but distinct segregation in branchial arches. *Development* **121,** 2569–2582.

Treanor, J. J., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F., Phillips, H. S., Goddard, A., Moore, M. W., Buj-Bello, A., Davis, A. M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C. E., and Rosenthal, A. (1996). Characterization of a multicomponent receptor for GDNF. *Nature* **382,** 80–83.

Trupp, M., Belluardo, N., Funakoshi, H., and Ibáñz, C. F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-α indicates multiple mechanisms of trophic actions in the adult rat CNS. *J. Neurosci.* **17,** 3554–3567.

Tucker, G. C., Ciment, G., and Thiery, J. P. (1986). Pathways of avian neural crest cell migration in the developing gut. *Dev. Biol.* **116,** 439–450.

Waddington, C. H. (1937). The determination of the auditory placode in the chick. *J. Exp. Biol.* **14,** 232–239.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., and Olson, L. (1997). Neurturin and glial cell line-derived neurotrophic factor receptor-β (GDNFR-β), novel proteins related to GDNF and GDNFR-α with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J. Neurosci.* **17,** 8506–8519.

Widenfalk, J., Tomac, A., Lindqvist, E., Hoffer, B., and Olson, L. (1998). GFRα-3, a protein related to GFRα-1, is expressed in developing peripheral neurons and ensheathing cells. *Eur. J. Neurosci.* **10,** 1508–1517.

Wilkinson, D. G. (1992). Whole-mount *in situ* hybridization of vertebrate embryos. In "In Situ Hybridization: A Practical Approach" (D. G. Wilkinson, Ed.), pp. 75–83. IRL Press, Oxford.

Williams, B. A., and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120,** 785–796.

Worby, C. A., Vega, Q. C., Zhao, Y., Chao, H. H.-J., Seasholtz, A. F., and Dixon, J. E. (1996). Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. *J. Biol. Chem.* **271,** 23619–23622.

Worby, C. A., Vega, Q. C., Chao, H. H.-J., Seasholtz, A. F., Thompson, R. C., and Dixon, J. E. (1998). Identification and characterization of GFRα-3, a novel co-receptor belonging to the glial cell line-derived neurotrophic receptor family. *J. Biol. Chem.* **273,** 3502–3508.

Wright, D. E., and Snider, W. D. (1996). Focal expression of glial cell line-derived neurotrophic factor in developing mouse limb bud. *Cell Tissue Res.* **286,** 209–217.

Yaginuma, H., Shiga, T., Homma, S., Ishihara, R., and Oppenheim, R. W. (1990). Identification of early developing axon projections from spinal interneurons in the chick embryo with a neuron specific β-tubulin antibody: Evidence for a new 'pioneer' pathway in the spinal cord. *Development* **108,** 705–716.

Yan, Q., Matheson, C., and Lopez, O. T. (1995). *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* **373,** 341–344.

Ye, W., Shimamura, K., Rubenstein, J. L. R., Hynes, M. A., and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93,** 755–766.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C., Hamada, H., and Noji, S. (1998). *Pitx2*, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94,** 299–305.

Yu, T., Scully, S., Yu, Y., Fox, G. M., Jing, S., and Zhou, R. (1998). Expression of GDNF family receptor components during development: Implications in the mechanisms of interaction. *J. Neurosci.* **18,** 4684–4696.

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